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The effects of silage inoculants on the fermentation and aerobic stability of legume-grass silage

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Abstract

The effects of silage additives homofermentative lactic acid bacteria (LAB) strains *Entrococcus faecium*, *Lactococcus lactis*, *Lactobacillus plantarum*; homo- and heterofermentative LAB strains *Lactobacillus plantarum*, *Entrococcus faecium* and *L. buchneri*; homofermentative LAB strains plus sodium benzoate; homofermentative LAB strains plus xylanase and homofermentative LAB strains plus xylanase and plus sodium nitrate on the fermentation and aerobic stability of legume-grass silages were studied under laboratory conditions. The second cut (265 g kg^{-1} dry matter) of a mixture of red clover and perennial ryegrass (1:1) stand was harvested with standard field equipment. After inoculants were added, the chopped forages were ensiled in a 3.0 litre anaerobic glass jars and compared with control silage prepared without an inoculant. The silages were opened after 90 days of ensiling and their chemical composition was measured. Silages were also assessed for aerobic stability.

Overall, microbial inoculants generally had a positive effect on grass-legume silage characteristics in terms of lower pH, ammonia-N concentrations, reduced DM losses and populations of yeasts and mould compared with control silages. The final pH and DM loss was lowest for silages inoculated with homofermentative LAB in combination with xylanase and for silages inoculated with homofermentative LAB in combination with xylanase and sodium nitrate. Homofermentative LAB in combination with *L. buchneri* or sodium benzoate shifted fermentation toward acetate and had a great effect on aerobic stability of slightly wilted red clover-ryegrass silages.

Key words: aerobic stability, fermentation, forage quality, grass and clover silage, inoculants.

Introduction

The mode of action of the additives applied to herbage during silage making can include limiting respiration or proteolysis by plant enzymes, manipulating fermentation, inhibiting the activity of clostridia and aerobic micro-organisms such as yeast and mould (Kung et al., 2003). Lactic acid is the most commonly identified organic acid that helps reduce the pH in the silage. Therefore, LAB are the primary type of bacteria utilized in most bacterial inoculants (McDonald et al., 1991; Filya et al., 2007). Many inoculants have selected strains of homofermentative LAB, such as *Lactobacillus plantarum*, *Enterococcus faecium*, *Lactococcus lactis*, and *Pediococcus* spp. LAB can result in a faster decrease in pH, lower final pH values, higher lactate:acetate ratios, lower ethanol and ammonia nitrogen, and a 1% to 2% improvement in DM recovery (Weinberg, Muck, 1996). Moreover, inoculants can provide substantial benefits by reducing the risk of the growth of other harmful spoilage organisms such as butyric acid bacteria including clostridia by reducing the pH (McDonald et al., 1991). Driehuis et al. (1999) showed that LAB affected activity of yeasts in two ways, firstly during anaerobic conditions, the survival of yeasts is reduced, and secondly, during the aerobic exposure, yeast growth is reduced. Inoculating forages at harvest with a heterofermentative LAB species *Lactobacillus buchneri* has improved the aerobic stability of the silages (Weinberg et al., 1999), because this organism converts lactic acid to acetic acid under anaerobic conditions (Oude Elferink et al., 2001) and this inhibits fungi and thus preserves silages susceptible to spoilage upon exposure to air (Filya et al., 2007). Recently, *L. buchneri* has been marketed in combination with homolactic acid bacteria, which are commonly added into silages to increase lactic acid production, rapidly drop pH, and decrease DM losses (Kung et al., 2003, Jaakkola et al., 2010).

Another alternative is to use additives containing other antimicrobial compounds. Such products containing sodium nitrate and sodium benzoate were used in the experiment reported in this article, because no long term studies on the effects of these combinations with bacteria strains on silage fermentation exist. Little information has been published on the use of potassium formate, sodium formate, and sodium benzoate for improving silage fermentation when applied alone to easy-to-

ensile forage (Cussen et al., 1995; Jaakkola et al., 2010) or difficult-to-ensile (Filya et al., 2007) forage. Nitrite, one of the intermediate products of nitrate degradation, is known for specific antimicrobial properties. Investigations of Lingvall and Lattemae (1999) demonstrated that the use of sodium nitrite and sodium benzoate can be effective. Silage additives sodium benzoate, sodium nitrite, hexamine and sodium propionate were efficient in improving fermentation and reducing nutrient losses and were sufficient to inhibit clostridial and yeast growth during the ensiling process, and resulted in stable silages at low dry matter levels (Knicky, Lingvall, 2004).

Some producers feel the inclusion of an enzyme in an inoculant can help the fermentation and digestibility of silages. Research conducted by Sheperd and Kung (1996) showed that treatment of maize (*Zea mays* L.) silage with an enzyme additive did not dramatically improve fermentation properties, but did reduce neutral detergent fibre (NDF) and acid detergent fibre (ADF) content and improved *in vitro* NDF digestion. Stokes (1992) reported that a combination of bacteria and enzymes was antagonistic to each other and did not improve silage fermentation, nutritional value, or animal performance when compared to either the enzyme or bacterial inoculant used alone, but there are no long term studies on the effects of this combination with bacteria on silage fermentation. Thus, the objectives of this study were to determine the effects of inoculants containing new combination of LAB strains with either no other components or with enzyme or/and chemical antimicrobial compounds on fermentation end-products and aerobic stability of legume-grass silages.

Materials and methods

Experiment was conducted in 2009–2010 at Institute of Animal Science Lithuanian Veterinary Academy according to the DLG (Deutsche Landwirtschafts-Gesellshaft e.V./ internationally acknowledged German Agricultural Society) Guidelines for the testing of silage additives and to the Guidelines on the assessment of safety and efficacy of silage additives, on a request from the Commission under Article 7(5) of Regulation (EC) No. 1831/2003 (EFSA-Q-2004-088). Adopted on 20 April 2006.

Test material. LAB strains rather than formulations were registered as silage additives in the EU. Therefore, trade names are not provided in Table 1.

Table 1. Blend of the bacterial strains or/and other components used as test material

Inoculant (product)	Bacterial strains or/and other components	Bacterial proportion %	Application rate cfu g^{-1} fresh forage
P1	*Lactobacillus plantarum* DSM 16568	20	250 000
	Entrococcus faecium NCIMB 11181	30	
	Lactobacillus buchneri (CCM 1819)	50	
P2	*Entrococcus faecium* NCIMB 11181	40	300 000
	Lactococcus lactis NCIMB 30117	30	
	Lactobacillus plantarum DSM 16568	30	
	Sodium benzoate at 400 g/ton forage		
P3	*Entrococcus faecium* NCIMB 11181	30	150 000
	Lactococcus lactis NCIMB 30117	30	
	Lactobacillus plantarum DSM 16568	40	
P4	*Entrococcus faecium* NCIMB 11181	30	250 000
	Lactococcus lactis NCIMB 30117	30	
	Lactobacillus plantarum DSM 16568	40	
	EC 3.2.1.8. Xylanase at 0.5 HEC/g forage		
P5	*Entrococcus faecium* NCIMB 11181	30	250 000
	Lactococcus lactis NCIMB 30117	30	
	Lactobacillus plantarum DSM 16568	40	
	EC 3.2.1.8. Xylanase at 0.5 HEC/g forage		
	Sodium nitrate at 1.3 kg ton forage		

cfu – colonyforming units

Inoculants were supplied by "Chr. Hansen A/S" (Denmark) as freeze-dried powders, sealed aluminium pouches clearly labelled. The test materials were stored at temperatures below 5ºC until used. Opened pouches were discarded after use.

Crop material and micro-silo preparation. A mixture of red clover (*Trifolium pretense* L.) cv. 'Arimaičiai' and perennial ryegrass (*Lolium perenne* L.) cv. 'Elena DS' (1:1 on fresh weight basis) at 2-year-old, second cut, at the early bloom stage of maturity of red clover was used in the present experiment. The forage was harvested in mid September. The crop was cut by a mower conditioner ("Kverneland Taarup 347"), wilted to a DM concentration of 265 g kg^{-1} and chopped by a forage harvester under farm conditions to 2–3 cm length. Chopped forage was transported in a polyethylene bag to the laboratory. Laboratory experiments started within 2 h from crop preparation. Five silage inoculants were used

in the experiment and 60 jars (micro-silos), 10 untreated and 50 inoculated were ensiled (Table 2).

The application rates of the inoculants were in accordance with the level of LAB in the inoculants as determined by manufacturer and in accordance with Table 1, column 4. The test materials were suspended in distilled water immediately prior to application. 2.00 g of each inoculant was diluted in 1000 ml chlorine-free H_2O targeting in a concentration of cells of 1.5 x 10^8 cfu ml^{-1} suspension (P3), 2.5 x 10^8 cfu ml^{-1} suspension (P1, P4 and P5), and 3.0 x 10^8 cfu ml^{-1} suspension (P2). Additionally, 400 g sodium benzoate for P2 and 1300 g sodium nitrate for P5 was diluted in the 1 litre suspension. 1.00 ml of each suspension was used per 1 kg forage (added 3 ml of chlorine-free water for uniform spraying). The same volume (4 ml g^{-1} of fresh forage) of chlorine-free water was used instead of the suspension in the control treatment (for spontaneous fermentation). Subsequently, the additives and water were sprayed into the fresh forage using a spray bottle and the forage was thoroughly mixed. The number of viable bacteria in each suspension used for inoculating the micro-silos were counted on DeMan-Rogosa-Sharpe (MRS) agar after incubation anaerobically at 37°C for 48 h (ISO 15214, Leuschner et al., 2003).

Table 2. Detailed study design

Factors	Number	Description
Experiments	1	Field 1 (red clover – ryegrass 1:1)
Replications	10	Micro-silo 1–10
Treatments	6	Untreated control and 5 different blends of the bacterial strains or/and other components as listed under test materials (in accordance with Table 1, column 4).
Total micro-silos	60	30 for testing fermentation quality, another 30 for testing aerobic stability after 90 days of ensiling

3-litre glass jars were used in the experiment. The density of forage in the silage was in compliance with DLG recommendations, 1 kg DM per 5 litre volume. Hereby, micro-silos were filled with 2.11–2.22 kg of fresh crop at DM concentration of 265 g kg^{-1}. Silos were closed immediately with caps, with a potential to vent gas, 30 min after being filled. Ensiling lasted for 90 days at a constant temperature of 20°C. At the end of the ensiling period, silages were subjected to chemical analysis and to aerobic stability test.

Sampling and analysis. Five representative samples (>500 g each) of fresh chopped forage were collected for subsequent chemical analysis. At the sampling time of silages on day 90 of the ensiling period, five micro-silos per treatment (30 silos) were weighed for determination of DM loss and subsequently opened and sampled to analyze the DM content, pH, fermentation products and ammonia-N. The remaining 30 micro-silos (5 from each treatment) were used for aerobic stability measurement. The DM content of forage and silage was determined by oven-drying at 105°C for 24 h. For the analysis of chemical composition of herbage, samples were oven-dried (1 h at 102°C and 48 h at 50°C) and then ground to pass a 1-mm sieve. Silage DM content was corrected for volatile alcohols and fatty acids during oven drying as described by Weissbach (2009). The total nitrogen was determined by Kjeldahl-AOAC 984.13. Crude protein (CP) content was calculated by multiplying the total nitrogen content by a factor of 6.25. The NDF and ADF concentrations were determined according to Van Soest et al. (1991) by using an Ankom^{200} fiber analyzer ("Ankom Technology", USA). Water-soluble carbohydrates (WSC) were determined using the anthrone reaction assay from the herbage or silage extracts obtained from steeping fresh herbage or silage in water. Ash concentration was determined by ashing the samples in a furnace at 600°C for 15 h. Buffering capacity of the forage was determined according to Playne and McDonald (1966), expressed as mEq of alkali required to change the pH from 4 to 6 per 1 kg of DM. Lactic acid, volatile fatty acid, alcohol, and ammonia N concentrations and pH were determined in silage extracts, prepared by adding 270 g of demineralized, deionized water to 30 g of silage and homogenizing for 5 min in a laboratory blender. Lactic acid, volatile fatty acids and alcohol concentrations were determined by gas-liquid chromatography. Gas-liquid chromatograph "GC-2010 Shimadzu" with wide-bore capillary column (Stabilwax®-DA 30 m, 0.53 mm, ID, 0.5 μm) was used according to Gas chromatography and Biochemistry analyzer official methods. Ammonia-N concentration was determined by direct distillation using the "Kjeltec Auto System 1030" (AOAC 941.04). The pH of silage was measured by using "Thermo Orion Posi-pHlo SympHony" electrode and "Thermo Orion 410" meter. Dry matter losses were estimated by measuring differences in silo weights after ensiling (on day 0 after ensiling) and at the end of the ensiling period (on day 90 after ensiling). Presence of *Clostridium perfringens* was quantified on reinforced clostridium agar Petri dishes incubated anaerobically at 37°C for 72 h (horizontal method for the enumeration of *Clostridium perfringens* – colony-count technique (ISO 7937:2004)). Amount of lactate-reducing yeasts and molds at the time of silage unloading was tested on extract-dextrose-chloramphenicol-agar-medium Petri dishes incubated aerobically at 25°C for 3 to 5 days (microbiology – general guidance for enumeration of yeasts and moulds – colony-count technique at 25°C (ISO 7954:1987(E)).

After opening the micro-silos, all silages were subjected to a 19-day aerobic stability test. A 1000 ± 10 g sample from each silo (five silos from each treatment) was loosely placed into a polystyrene box according to recommendations from DLG and allowed to aerobically deteriorate at constant room temperature (≈20ºC). The top and bottom of the boxes contained a 2-cm-diameter hole to allow air to enter and CO_2 to leave. A transducer was placed in the centre of the silage mass through a hole in the cover of the box, which exposed the silage to air. These silages were not disturbed during the period of recording the temperatures. Ambient temperature and the temperature of each silage was recorded every 6 h by a data logger. Ambient room temperature was measured by using an empty control box. Aerobic stability of silages was examined by calculating the differences between silage temperature and ambient room temperature adjusted for base ambient temperature. Aerobic stability was defined as the number of hours the silage remained stable before rising more than 3°C above the ambient temperature (Moran et al., 1996).

Statistical analyses. Five replications were used per additive treatment. Silage composition data were subjected to one-way analysis of variance for a 6 (additive) factorial arrangement of treatments within a randomized complete block design by using *Proc GLM* of SAS (Statistical Analysis System SAS®, version 8.02, 2000). Aerobic stability data for each herbage type were analyzed separately by one-way analysis of variance in a randomized complete block design and where temperatures were treated as repeated measurements. Significance was declared at $P < 0.05$.

Results

The same forage was used for all treatments. The chemical and microbiological composition of the fresh forage is presented in Table 3. Analyses from the fresh crop revealed medium concentration of WSC and high concentration of crude protein. The buffering capacity of forages was medium. The crop had a medium count of yeast and moulds.

Table 3. Chemical composition of a mixture of red clover and perennial ryegrass (1:1) at ensiling

Item	n	Mean	Std
DM, g kg^{-1}	5	265.1	0.553
Crude protein, g kg^{-1} DM	5	174.4	3.061
Crude ash, g kg^{-1} DM	5	112.2	2.129
WSC, g kg^{-1} DM	5	89.5	4.592
ADF, g kg^{-1} DM	5	286.8	6.723
NDF, g kg^{-1} DM	5	360.6	11.908
Buffering capacity, mEq kg^{-1} DM	5	341.9	46.313
OMD, %	5	68.8	1.789
pH	5	6.6	0.031
Clostridia spores, log cfu g^{-1} FM	5	<1.0	0
Yeast, log cfu g^{-1} FM	5	3.8	1.228
Moulds, log cfu g^{-1} FM	5	4.9	0.352

Note. DM – dry matter, FM – fresh matter, WSC – water-soluble carbohydrate, OMD – organic matter digestibility, ADF – acid detergent fibre, NDF – neutral detergent fibre, cfu – colony forming units.

Due to unfavourable weather conditions (15–16/09/2009 – 12.5 hours of day light, low ambient temperature (+13°C) and high relative humidity (73%), wilting of herbage was incomplete and herbage was wilted to DM concentration of 265 g kg^{-1}.

The results of the chemical analyses of the silages after 90 days of ensiling are presented in Table 4.

Inoculation resulted in higher ($P < 0.05$) DM concentrations in all examined silages in comparison with the control. The highest DM concentrations were observed in silages treated with inoculants P4 and P5. Silage made without additive was poorly fermented, as was indicated by its relatively high pH, ammonia-N and butyric concentration and DM loss and low lactic acid concentration. Upon examination of pooled experiment data from all six treatments, large effects of the inoculants on the fermentation indicators were detected. Silages inoculated with all 5 inoculants showed a lower ($P < 0.05$) final pH as well as lower ($P < 0.05$) DM loss than uninoculated silages. The final pH and DM loss was lowest for silages inoculated with homofermentative LAB in combination with xylanase (P4) and for silages inoculated with homofermentative LAB in combination with xylanase and sodium nitrate (P5).

Inoculation significantly increased lactic acid and acetic acid concentrations, and significantly decreased butyric acid, alcohols and ammonia-N concentrations compared with untreated silages. Among inoculated silages the largest ($P < 0.05$) increase of lactic acid was observed in silages treated with products P3, P4 and P5, and the highest ($P < 0.05$) increase in acetic acid was detected in silages treated with inoculants P1, P2 and P4. Silages inoculated with P4 had a lower ammonia-N concentration ($P < 0.05$) but higher ($P < 0.05$) alcohol concentration than silages treated with other inoculants. Silage inoculated with P1 had significantly higher con-

centration of propionic acid and numerically higher concentration of butyric acid than other inoculated silages. Inoculated silages had significantly lower ($P < 0.05$) yeast and mold count than uninoculated silage, whereas no differences were observed between products used for silages inoculation.

Table 4. Comparison of the composition and DM loss of silages after 90 days of ensiling with added blends of bacterial strains or/and other components as silage inoculants

Item	No additive	P1	P2	P3	P4	P5	$LSD_{0.05}$	EMS
DM, g kg^{-1}	238^{c}	249^{b}	251^{b,a}	249^{b}	254^{a}	252^{b,a}	3.172	0.581
DM loss, %	12.3^{a}	6.7^{b}	5.7^{c,b}	6.4^{c,b}	5.0^{c}	5.5^{c,b}	1.517	1.322
Ammonia-N, % of total N	9.2^{a}	5.1^{b}	4.4^{c}	4.6^{c,b}	3.7^{d}	4.2^{c,d}	0.616	0.218
pH	5.55^{a}	4.71^{b}	4.35^{c}	4.32^{c}	4.23^{d}	4.26^{d}	0.042	0.001
Lactic acid, g kg^{-1} DM	13.9^{d}	37.7^{c}	57.1^{b}	62.6^{b,a}	63.7^{b,a}	71.0^{a}	9.913	56.456
Acetic acid, g kg^{-1} DM	18.2^{b}	32.1^{a}	32.6^{a}	23.7^{b}	31.0^{a}	20.4^{b}	6.792	26.506
Butyric acid, g kg^{-1} DM	37.5^{a}	1.8^{b}	0.3^{b}	0.9^{b}	0.4^{b}	0.2^{b}	2.334	3.129
Propionic acid, g kg^{-1} DM	0.7^{b}	1.9^{a}	0.3^{c,b}	0.4^{c,b}	0.3^{c,b}	0.2^{c}	0.449	0.116
Alcohols, g kg^{-1} DM	14.7^{a}	5.7^{c,b}	3.8^{c,d}	5.0^{c,b,d}	6.1^{b}	3.1^{d}	1.951	2.187
Clostridia spores, log cfu g^{-1} FM	0.99^{a}	0.99^{a}	0.99^{a}	0.99^{a}	0.99^{a}	0.99^{a}	0.004	0.000
Yeast, log cfu g^{-1} FM	3.18^{a}	1.31^{b}	1.36^{b}	1.51^{b}	1.62^{b}	1.54^{b}	0.614	0.221
Moulds, log cfu g^{-1} FM	3.00^{a}	1.30^{b}	1.40^{b}	1.44^{b}	1.58^{b}	1.42^{b}	0.391	0.090
Aerobic stability, h	192^{d}	>450^{a}	366^{b}	258^{c}	258^{c,b}	258^{c}	4.221	21.243

Note. DM and calculated DM losses are corrected for volatiles; $^{a, b, c, d, e}$ – means with different superscript letters in a line indicate significant differences of $P < 0.05$; DM – dry matter, FM – fresh matter, cfu – colony forming units.

Irrespective of treatment, all of the micro-silo silages were stable for 156 h (6.5 days) after the silos were opened and aerated (Fig.).

During aerobic exposure, untreated silage had a temperature rise of more than 3ºC above the ambient after 192 h and reached temperatures of more than 7ºC above ambient temperature after 318 h. Silages treated with (P1) remained stable during the full aerobic stability measurement period and had temperature rise only 2ºC above the ambient. Silages inoculated with (P2) had a temperature rise of more than 3ºC above the ambient after 366 h. Silages inoculated with P3, P4 and P5 had a temperature rise of more than 3ºC above the ambient after 258 h. However, silages P3 and P5 reached a temperature of more than 6ºC above ambient temperature after 318 h. Silages inoculated with P4 reached a temperature of less than 4ºC above the ambient temperature. Consequently, inoculant P1 increased silage aerobic stability dramatically, inoculant P2 increased ($P < 0.05$) aerobic stability by 174 h (7 days) inoculants P3, P4, and P5 increased ($P < 0.05$) aerobic stability by 66 h (2.7 days) in comparison with untreated silages.

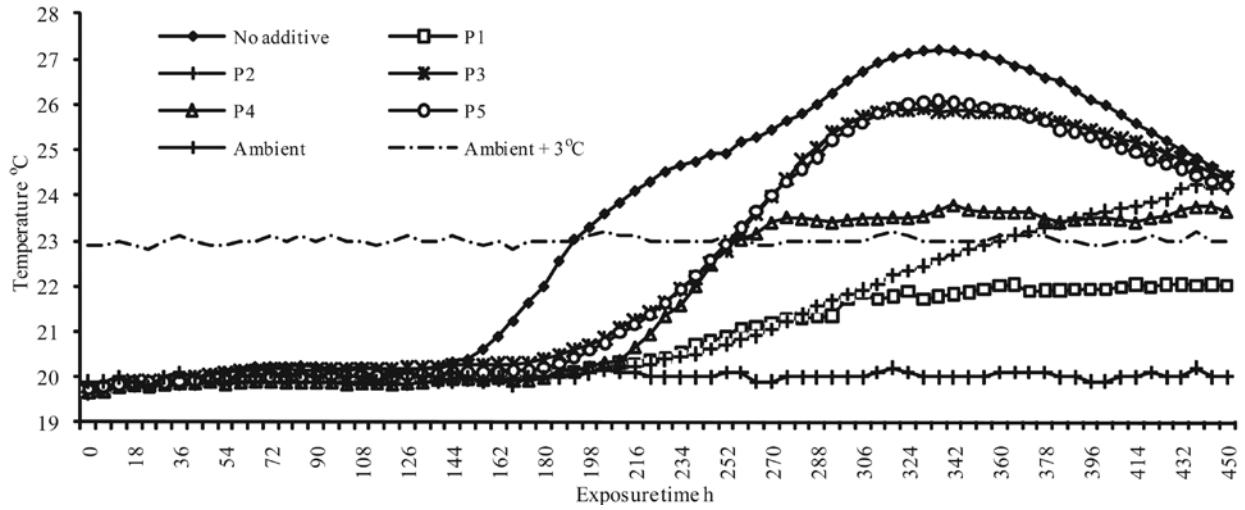

Figure. Aerobic stability of silages after 90 days of ensiling in micro-silos untreated or inoculated with bacteria blends or/and other components

Discussion

The chemical and microbial composition of fresh forage crop determines its ensilability, which is regarded to be a precondition for successful ensiling. Thus, in the present experiment the low DM content (265 g kg^{-1}) in combination with medium concentration (89 kg^{-1} DM) of WSC and high concentration (174 kg^{-1} DM) of crude protein of the forage indicates it was difficult to ensile as suggested also by McDonald et al. (1991). Therefore, the trial provided a great challenge for the inoculants to improve silage fermentation.

In the present experiment, untreated silage was poorly preserved as evidenced by high pH and high butyric acid and ammonia-N concentrations, all typical of clostridial silages. A lower pH value should be reached for moist forage to prevent clostridial activity (Pahlow et al., 2003). It is probable that fermentable carbohydrates might have been depleted by that time and lactic acid fermentation ceased and butyric acid fermentation and ammonia N formation continued (Pursiainen, Tuori, 2008). Silages treated with examined inoculations were characterized by higher concentrations of DM compared with the untreated silages and may be explained by the addition of DM to the silages with additives as well as delays in the fermentation processes in the untreated silages, which caused DM losses (McDonald et al., 1991). This also finds confirmation in literature (Driehuis et al., 2001). All the additives improved the fermentation quality compared with the control silage. Inoculation with tested products significantly reduced pH, butyric acid, ammonia-N, alcohol concentrations and DM loss and significantly increased lactic acid concentrations, relative to the untreated silages. The beneficial effect of the inoculants was related to a more rapid and higher acidification that probably reduced the activity of plant enzymes and proteolysis. Lower values of pH in the inoculated silages compared to those of the untreated silages indicate that fermentation was initiated effectively by added LAB. Filya et al. (2007) concluded that main effect of silage inoculants was the increased production of lactic acid connected with significant reduction of pH value and minimised dry matter losses. Weinberg and Muck (1996) stated that LAB can result in a in lower pH values, higher lactic acid concentration, lower ethanol and ammonia nitrogen content, and about 2 percentage units improvement in DM recovery. Due to an improved fermentation and conservation effect a significantly lower DM loss was achieved in treated silages in our experiment. Comparable effects have been found for inoculant treatment of grass silages with low DM content (Cussen et al., 1995) and lucerne silages (Jones et al., 1992). The applied additives reduced ($P < 0.05$) the numbers of yeast cells and mould fungi in comparison with the control. Kleinschmit et al. (2005) found reduced fungal populations in silages treated with LAB or LAB-enzymatic additives. Knicky and Lingvall (2004) obtained reduction of yeast growth by application of sodium benzoate and sodium nitrite in both low and high DM red clover and timothy silages. Mixtures used in this experiment suppressed yeast growth and was reflected in the concentration of alcohols, generally correlated to yeast activity in silages.

Among the five additives used for inoculation there were significant differences in fermentation products. Silages inoculated with *L. buchneri* plus homofermentative LAB (P1) had significantly higher pH and propionic acid concentration, significantly lower lactic acid concentration and lactic to acetic acid ratio when compared with other inoculants used in the present experiment. The reduction in the lactic acid with *L. buchneri* was reported by Oude Elferink et al. (2001). Driehuis et al. (2001) also reported a marked reduction in lactic acid concentration and an increase in propionic acid concentration in silages treated with *L. buchneri*. The silages inoculated with homofermentative LAB in combination with Xylanase (P4) and homofermentative LAB in combination with Xylanase and sodium nitrate (P5) had lowest pH when compared with other treatments. The highest lactic acid concentrations and lactic to acetic acid ratios were observed in silages inoculated with P5, P4 and P3. In agreement with Adesogan et al. (2004) treatment of bermudagrass, which is low in sugars, with an enzyme-inoculant blend improved the fermentation, but contradictory results exist (Mandevbu et al., 1999). Several studies have also demonstrated that enzyme application especially in the presence of microbial inoculants improves the fermentation of cool season grasses (Beuvink, Spoelstra, 1994) and lucerne (Beuvink, Spoelstra, 1994; Nadeau et al., 2000) silage. One key factor that influences proteolysis in the silo is the speed of pH decline (Kung et al., 2003). The faster acidification of the silage and higher lactic acid concentration lead to a reduction in proteolysis (Cussen et al., 1995). The fermentation processes possibly culminate during a shorter period in the P4 and P5 inoculated silages resulting in less proteolysis of plant proteins and ammonia-N concentrations. Multiplication of clostridia and enterobacteria, which are responsible for protein degradation resulting in the formation of ammonia, stops at pH 4.5 (Pahlow et al., 2003). The positive effects of LAB inoculants on nitrogen fractions can be explained either by domination of the fermentation resulting in the rapid achievement of a low pH or via the low proteolytic activity of the strains (Winters et al., 2000). The P4 silage had the highest alcohols content. Kleinschmit et al. (2005) reported a decline of ethanol in silages treated with bacterial-enzyme preparations, whereas inoculants containing organic acids were found to increase the content of alcohols.

Irrespective of treatment, all of the micro-silo ensiled red clover-ryegrass (1:1) silages were stable for 156 h (6.5 d) after the silos were opened and aerated. Certain plant characteristics could make a positive contribution towards limiting aerobic spoilage in the silo, and legume silages (red clover and lucerne) are more stable than maize or grass silage (Driehuis et al., 1999). The data presented in this paper show that inoculation with *L. buchneri* in combination with homofermentative LAB (P1) as well as inoculation with homofermentative LAB in combination with sodium benzoate (P2) increase the

fastness of slight wilted red clover-ryegrass to aerobic deterioration. The improvement of aerobic stability was observed in a timothy-meadow fescue silage by using *L. buchneri* or sodium benzoate in combination with *L. plantarum* (Jaakkola et al., 2010). Danner et al. (2003) investigated the effect of different compounds formed by heterofermentative lactic acid bacteria on aerobic stability of silage and concluded that aerobic stability was directly correlated to the amount of acetic acid present in silages. The amount of undissociated acetic acid has been identified as the most important factor to consider when attempting inhibiting yeast growth in silages. Weinberg et al. (1999) investigated several homofermentative lactic acid bacteria for their potential effect on aerobic stability when used as silage inoculants. However, inoculating silages with lactic acid bacteria has not always resulted in silage with good aerobic stability (Kung et al., 2003). Other studies (Danner et al., 2003) provide more definitive evidence for the existence of certain LAB strains able to inhibit yeast and molds growth and to improve aerobic stability.

Conclusions

1. Lactic acid bacteria (LAB) inoculants were effective in improving fermentation quality and had a positive effect on red clover-ryegrass silages characteristics in terms of lower pH, ammonia-N concentrations, reduced DM losses and populations of yeasts and mould compared with control silage.

2. Homofermentative LAB and homofermentative LAB in combination with xylanase shifted fermentation toward lactate. Homofermentative LAB in combination with *L. buchneri* or sodium benzoate shifted fermentation toward acetate and had a great effect on aerobic stability of slightly wilted red clover-ryegrass silages.

3. Inoculation with homofermentative LAB in combination with xylanase resulted in lowest protein degradation as evidenced by lower ammonia-N concentrations and lowest DM losses.

4. Further investigations are required to find the optimal combinations of homo- and heterofermentative LAB or chemical salts and the results obtained on the laboratory scale need to be verified in more practical conditions.

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Biologinių priedų įtaka pupinių bei miglinių žolių siloso fermentacijos rodikliams ir aerobiniam stabilumui

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Santrauka

Tyrimų tikslas – nustatyti biologinių silosavimo priedų (inokuliantų) poveikį pupinių ir miglinių žolių siloso kokybiniams rodikliams. Silosavimo metu naudotas homofermentatyvinių pieno rūgšties bakterijų mišinys: *Entrococcus faecium*, *Lactococcus lactis*, *Lactobacillus plantarum*; homofermentatyvinių ir heterofermentatyvinių pieno rūgšties bakterijų mišinys *Lactobacillus plantarum*, *Entrococcus faecium* ir *L. buchneri*; homofermentatyvinių pieno rūgšties bakterijų mišinys ir natrio benzoatas; homofermentatyvinių pieno rūgšties bakterijų mišinys bei fermentas ksilanazė ir homofermentatyvinių pieno rūgšties bakterijų mišinys bei fermentas ksilanazė ir natrio nitratas. Silosas buvo pagamintas laboratorinėmis sąlygomis iš antros pjūties raudonųjų dobilų ir daugiamečių svidrių mišinio (1:1), pavytinto iki 265 g kg^{-1} sausųjų medžiagų. Žolė nupjauta ir susmulkinta naudojant standartinę žolių dorojimo ir smulkinimo techniką. Susmulkinta žaliava silosuota be priedų arba apdorojus vienu iš inokuliantų, 3 litrų talpos hermetiškai uždaromuose laboratoriniuose induose. Laboratorinės siloso talpos buvo atidarytos nuo silosavimo praėjus 90 dienų ir paimti mėginiai jų cheminei sudėčiai bei aerobiniam stabilumui nustatyti. Visi tyrimų metu naudoti silosavimo priedai turėjo teigiamos įtakos siloso kokybei ir sumažino siloso pH rodiklį, amoniakinio azoto koncentraciją, sausųjų medžiagų nuostolius ir mielių bei pelėsių populiaciją, palyginti su silosu be priedų. Žemiausias pH rodiklis ir mažiausi sausųjų medžiagų nuostoliai buvo siloso, pagaminto su homofermentatyvinių pieno rūgšties bakterijų mišiniu kartu su fermentu ksilanaze ir natrio nitratu. Homofermentatyvinės pieno rūgšties bakterijos kartu su heterofermentatyvine *L. buchneri* silose padidino acto rūgšties kiekį ir gerokai pagerino jo aerobinį stabilumą.

Reikšminiai žodžiai: aerobinis stabilumas, fermentacija, dobilų ir svidrių silosas, pašaro kokybė, inokuliantas.